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Original communication

Hypothalamic transcript profiling in hypothermia using SuperSAGE

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ABSTRACT

Understanding of molecular mechanisms underlying hypothermia is of primary importance in devising strategies to diagnose hypothermia. We investigated the hypothalamic transriptome in hypothermia. For transcriptomic analyses, SuperSAGE, an improved method of serial analysis of gene expression, was used. Totally, 62,208 and 54,084 tags were collected from the hypothalami of normal and hypothermia, respectively. And 367 transcripts were differentially expressed at a statistically significant level. That is, 157 and 210 transcripts among them were expressed at a higher level in normal and hypothermic hypothalami. Results obtained by SuperSAGE and quantitative PCR were consistent in 6 selected genes, although levels of differences detected by the 2 methods were not exactly the same. mRNA expressions in the hypothalamus were considered to be useful for hypothermic diagnosis. Various methods have been applied for gene expression analyses and biomarker detections. However in forensic pathology, SuperSAGE would be a promising method, especially in gene discoveries and transcriptomic analyses.

1. Introduction

Compared with the broad temperature range for terrestrial climates, the human body temperature is usually regulated within a very narrow range.[1] Hypothermia develops when this adaptive mechanism is overwhelmed.[2] Cold diuresis and hypoglycemia occur in the early phase of hypothermia,[3,4] and may be accompanied by frostbite; hemorrhagic pancreatitis; erosions and hemorrhages of the gastric mucosa, ileum, and colon; bronchopneumonia; acute tubular necrosis; cardiac muscle degeneration.[4,5] However, hypothermia is difficult to diagnose, since so far only general indications of stress have been observed, not specific changes in organs caused by cold. The diagnosis must be made by exclusion and by relying on anamnetic information. Therefore, the search for more reliable necropsy signs is important, and biochemical analyses could give more valuable information. The usefulness of fatty degeneration and heat shock protein 70 in kidney for hypothermic diagnosis[6,7] has been suggested. The effects of cold may bring on mental sluggishness and confusion. Victims of hypothermia are often found undressed,[8] which is explained by thermal hallucination caused by paralysis of the thermal regulatory metabolism.[9] It is also reported that the human body regulates temperature and physiologic responses to cold by way of the hypothalamus.[2]

For biomarker analyses, various methods have been applied. However, conventional techniques such as northern blotting, and RT-PCR allow the evaluation of only a limited number of genes at one time. In addition, differential screening or differential display techniques are not very accurate in quantitatively monitoring gene expression.[10] Therefore, high-throughput, quantitative methods for gene expression analysis are required. "SuperSAGE", an improved serial analysis of gene expression (SAGE) variant involving the endonuclease EcoP15I, has been reported as such a tool.[11–13]

The aims of this study are to analyzed the hypothalamic transcript for the diagnosis of hypothermia, and to introduce "SuperSAGE" as a research tool for forensic pathology.

2. Materials and methods

2.1. Tissue samples

The water bath model[14] was adopted. Seven-week-old male Wister rats, weighing 315.4 ± 26.6 g, were housed under controlled lighting (lights on at 7:00 am and off at 7:00 pm) and given free access to food and water. The rats were anesthetized by sevoflurane inhalation, and confined in a metallic restraint cage which was kept in a water bath at 4 °C, so that the neck was immersed. The animals were sacrificed by continuous cold exposure to the cold water

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(47.3 ± 10.9 min). The right hypothalamus was resected ($n = 10$ for SuperSAGE, $n = 10$ for quantitative PCR) as described by Glowinski and Iversen.[15]

As a control, specimens from intact rats were examined simultaneously ($n = 10$ for SuperSAGE, $n = 10$ for quantitative PCR).

2.2. Extraction of total RNA and reverse transcription

Total RNA was extracted from the tissues by Ribopure (Applied Biosystems, Carlsbad, CA). The quality of the RNA was evaluated using electrophoresis on a 1% agarose gel and staining with ethidium bromide.

2.3. SuperSAGE

Ten intact samples were pooled into 1 pooled control sample, and 10 hypothermia model samples were pooled into 1 pooled hypothermic sample, to eliminate any individual variations, and to extract sufficient quantities of mRNA. From total RNA, 5 μg of mRNA were isolated using the illustra mRNA Purification Kit (GE Healthcare, Little Chalfont, UK). This mRNA was reverse-transcribed (SuperScript Double-stranded cDNA synthesis kit, Invitrogen, Carlsbad, CA) to generate single-stranded cDNA by using reverse transcription-primer with the sequence 5′-biotin-CTGATCTAGAGGTACCGGATCCCAGCAGTTTTTTTTTTTTTTT-3′, containing the 5′-CAGCAG-3′ recognition site of the restriction enzyme EcoP15I. The product was converted to double-stranded cDNA and digested with NlaIII (New England BioLabs, Ipswich, MA). The cDNA was divided into two portions in separate tubes, respectively. Streptavidin Magnesphere Paramagnetic Particles (Promega, Madison, WI) was added to the digestion reaction and the 3′-end fragment of the cDNAs were bound to streptavidin magnetic beads. Two sets of linkers (linker 1 and 2 for control, linker 3 and 4 for hypothermia) were prepared by annealing commercially synthesized oligonucleotides (Qiagen, Valencia, CA).

linker-1,

5′-TTTGGATTTGCTGGTGCAGTACAAGAAGGCTTAATA-CAGCAGCATG-3′
5′-CTGCTGTATTAAGCCTTCTTGTACTGCACCAGCAAATCCAAA-3′-NH_2

linker-2,

5′-TTTCTGCTCGAATTCAAGCTTCTATCGATGCACGCAGCAGCATG-3′
5′-CTGCTGCGTGCATCGATAGAAGCTTGAATTCGAGCAGAAA-3′-NH_2

linker-3,

5′-TTTGGATTTGCTGGTGCAGTACAACTACGTCTAGTA-CAGCAGCATG-3′
5′-CTGCTGTACTAGACGTAGTTGTACTGCACCAGCAAATCCAAA-3′-NH_2

linker-4,

5′-TTTCTGCTCGAATTCAAGCTTCTATCGATGCACACAGCAGCATG-3′
5′-CTGCTGTGTGCATCGATAGAAGCTTGAATTCGAGCAGAAA-3′-NH_2

The unblocked 5′ termini of linkers were phosphorylated by T4 polynucleotide kinase (TaKaRa, Otsu, Japan). The linkers harbor the EcoP15I recognition sequence (5′-CAGCAG-3′). To each of 4 tubes containing cDNA bound to magnet beads, linker-1 or -2, and linker-3 or -4 were added and ligated to the cDNA ends by T4 DNA ligase (Invitrogen, Carlsbad, CA). Linker-1-tag and linker-2-tag fragments, and linker-3-tag and linker-4-tag fragments were mixed, respectively. EcoP15I recognizes the sequence 5′-CAGCAG-3′ and cleaves the DNA 25 nt (in one strand) and 27 nt (in the other strand) downstream of the recognition site leaving a 5′ overhang of two bases. Two inversely oriented recognition sites (5′-CAGCAG-N(i)-CTGCTG-3′) are essential for efficient cleavage.[16] Linker-ligated cDNA on the magnetic beads was digested with EcoP15I (New England BioLabs, Ipswich, MA). Released fragments were separated on PAGE, and the "linker-tag" fragments of 66, 68 bp (linker, 40 or 42 bp; tag, 26 bp) were visualized by FITC fluorescence on a UV transilluminator and collected from the gel. The fragments were blunt-ended by filling-in with Blunting high KOD DNA polymerase (Toyobo, Osaka, Japan), and ligated to each other (DNA ligation kit ligation high, Toyobo, Osaka, Japan). The resulting ditags were amplified by PCR (TaKaRa Ex Taq, TaKaRa, Otsu, Japan) with primers.

ditag primer 1, 5′-CAAGAAGGCTTAATACAGCAGCA-3′;
ditag primer 2, 5′-CTATCGATGCACGCAGCAGCA-3′.
ditag primer 3, 5′-CAACTACGTCTAGTACAGCAGCA-3′;
ditag primer 4, 5′-CTATCGATGCACACAGCAGCA-3′.

Ditag PCR products were separated on PAGE and the fragment of 94 bp was isolated from the gel. The ditags were directly sequenced with 454 sequencing systems (454 Life Sciences, Branford, CT). For tag extractions, sequence data were analyzed with programs for SuperSAGE.[13] The proportion in normal and hypothermic libraries was compared by χ^2 analysis,[17] and P values of 0.05 or less were considered statistically significant. For annotations of SuperSAGE tags, sequence files were analyzed by resources of National Center for Biotechnology SAGE Web page (http://www.ncbi.nlm.nih.gov/SAGE).

2.4. Quantitative PCR

To validate selected aspects of SuperSAGE results, 3 abundantly expressed genes in the control rats (neuroprotective protein 8, Purkinje cell protein 4, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide), and 3 genes in the hypothermic rat (heat shock protein 8, reticulon 1, solute carrier family 6, member 1) were selected for measurements by quantitative PCR.

2.4.1. Reverse transcription

cDNA was synthesized with High Capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Carlsbad, CA), following the manufacturer's instructions.

2.4.2. Positive control preparation

TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) was adopted. The following primers and probe were used (neuroprotective protein 8: Rn00595851_m1, Purkinje cell protein 4: Rn00564515_m1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide: Rn00755085_m1, heat shock protein 8: Rn00821195_g1, reticulon 1: Rn00587875_m1, solute carrier family 6, member 1: Rn00577652_m1). As an internal standard, β-actin was employed. The β-actin primers and probe were also given by TaqMan Gene Expression Assays (Rn00667869_m1, Applied Biosystems, Carlsbad, CA). PCR amplification was performed with Phusion high fidelity DNA polymerase (New England Biolabs, Ipswich, MA). After initial denaturation at 98 °C for 30 s, amplification consisting of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s was

Table 1
Summary of SuperSAGE analysis in hypothalamus.

Sample	Total number of tags	Number of unique tags
Control	62,208	26,988
Hypothermia	54,084	23,990

performed for 35 cycles. The amplified products were identified using electrophoresis on a 3.5% agarose gel and staining with ethidium bromide. Subsequently, the DNA were purified using QIAEX II gel extraction kit (QIAGEN, Valencia, CA). Purified DNA were ligated into the plasmids (3519 bp) and subcloned with DH 5α *Escherichia coli* using Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA were isolated using PI-200 DNA automatic isolation system (Kurabou, Osaka, Japan), and DNA in the plasmid was confirmed by cycle sequence.

Table 2
The 50 most abundantly expressed genes in control rats.

Tag sequence	Count of tags		Accession no	Corresponding gene
	Control	Hypothermia		
CATGCTGCTGTATTAAGCCTTCTTGC	442	71		No match
CATGCTAGTCTTTGTGCACACAGAAT	126	82	BC058150	Ribosomal protein S29
CATGTTGGTGTCCGCAAGGGTAGAGA	125	80		No match
CATGCTGCTGCGTGCATCGATAGCAA	109	13		No match
CATGTCTTCGTGACTGTGACCTCACT	99	56	EU000468	Neuroprotective protein 8
CATGCTGCTGCGTGCATCGATAGCTA	97	11		No match
CATGAACAGAAACCCATAGTATTTAT	92	34	NM_013002	Purkinje cell protein 4
CATGATGGGTGGTTATGAAATGCCAG	85	47	NM_013052	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide
CATGGTGATGTGGCCTCAGGACTCCC	72	40	NM_013015	Prostaglandin D2 synthase
CATGAGCGGGAGGTGCTGCACAAGGC	62	20	NM_024346	Stathmin-like 3
CATGCTGCTGCGTACAGCGATAGCAA	51	28		No match
CATGTATGACTTAATAAATCCTTGAA	47	16		No match
CATGGGATTCGGTCTCTTCGACTAAG	44	23	BC062028	Acidic ribosomal phosphoprotein P0
CATGGATAAAACCAGACCATAAGGAA	44	18	NM_053515	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 (Slc25a4), nuclear gene encoding mitochondrial protein
CATGAATTTGATATAATGCAAGTGAC	41	20		No match
CATGACAAAAGCACTTTGGGGAATGA	39	19	BC093596	Proteolipid protein 1
CATGGAAATAACGGTGAGATTCCAGC	34	12	BC063161	Phosphoglycerate kinase 1
CATGAACCCGCTTCCGACTGGCTATG	33	14	NM_012814	Cytochrome c oxidase, subunit VIa, Polypeptide 1 (Cox6a1), nuclear gene encoding mitochondrial protein
CATGGTGATGGCCACAGTTAAGAAAG	29	12	NM_001007599	Ribosomal protein L23
CATGTAGGAGACTGGTGATGTGTTGA	29	10	AJ230633	PKCd gene encoding protein kinase C delta
CATGGAGGAGCCGCATTACAGTTCTG	29	6	BC089963	Cell cycle exit and neuronal differentiation 1
CATGCATTGCGTGGTTGTAATCAAAG	27	8	BC083818	Eukaryotic translation initiation factor 4H
CATGCTGCTGTATTAAGCCTTCTTGT	25	2		No match
CATGGAGGCTTTGCAGGCTGGTGCAG	24	9	BC085120	Heat shock protein 90, alpha, class A member 1
CATGAATCCTGTGGAGCATCCCTTTG	24	8	BC126063	Ribosomal protein L8
CATGCGCTGGTTCCAGCAGAAGTATG	23	8	BC097372	Ribosomal protein L11
CATGGGGTACAGCCCAGAGGGAGGGG	23	8	BC107454	FK506 binding protein 8, 38 kDa
CATGGCGGGGTCGCCGCTGCTCTGCG	23	7	AF181561	proSAAS
CATGCAAGGTGACAGGCCGCTGTGGC	23	7		No match
CATGGGAAGGTGGCCCAAACCCAAGA	22	6	X55572.1	Apo D
CATGGGGGAAATCGCCAGCTTCGATA	21	8	NM_021261	Thymosin, beta 10
CATGTGGCCCAACACAAAACAGGAAA	21	8	NM_001108533	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2
CATGTAGACTCTCACAGCCCAGAGCT	20	7	AY255791	NDRG4-A1
CATGGGAGATACCTGTTCAAAGAATG	19	5		No match
CATGGCCTCCTCTGGCTGAGCAGAGC	18	4	NM_031351	Attractin
CATGCGTAGTGCTTGTGAGAAGAGGC	18	4	NM_001108888	Receptor accessory protein 5
CATGGCCTCCACGTAATTATTGGCTC	17	6	AF504920	ATPase synthase subunit 6 mRNA, partial cds; cytochrome oxidase subunit III mRNA, complete cds; tRNA-Gly gene, complete sequence; and NADH dehydrogenase subunit III mRNA, complete cds; mitochondrial genes for mitochondrial products
CATGTAGCTGTAATGGGGGGCGGGGG	17	5	NM_012504	ATPase, Na+/K+ transporting, alpha 1 polypeptide
CATGGCGAAACTCAGAGAGTAGTGTG	17	5		No match
CATGTGAATATTTTGCTGGGTTACAC	16	5		No match
CATGGCCTCGTCCCAGTAACTGTGTG	16	4	NM_001106242	Eukaryotic translation initiation factor 3, subunit K
CATGCTGCTGTATTAAGCCTTCTTGA	16	4		No match
CATGTTAATGGCGAAAGAATGGAAGC	15	4	NM_054006	Cold shock domain containing E1, RNA binding
CATGACATTTCTCTGCAAAGAACTCT	15	4		No match
CATGAGGAGAAAGGATCCAGGCTTTG	15	4	BC167055	Vacuolar protein sorting 29
CATGGGAAGTGAAAGATACTTATGAG	15	4	NM_031831	Reticulon 4
CATGTTTAGTGGTATCTGACGACAGG	15	1		No match
CATGGGATTTGTTCTAATAAATCATT	14	4		No match
CATGAGTAAGTGGACTGTAGTCGGAC	14	4	NM_001003401	Ectodermal-neural cortex 1
CATGCCCGTGTGCTCATCCGCCAACG	14	4	BC060560	Ribosomal protein S9

2.4.3. Real-time quantitative PCR

Real-time quantitative PCR was performed using PRISM 7500 sequence detector (Applied Biosystems, Carlsbad, CA). A 50 μl reaction mixture containing TaqMan gene expression Master Mix (Applied Biosystems, Carlsbad, CA) was used, and thermal cycler conditions given in the manufacturer's instruction were followed. All samples were analyzed in triplicate. Expressions between control and hypothermic hypothalami were analyzed using the *t* test, and *P* values of 0.05 or less were considered statistically significant.

The research described in this report was conducted in accordance with the guidelines for animal experimentation, Iwate Medical University.

3. Result

3.1. General results and analysis of superSAGE tags

A SuperSAGE library was constructed from mRNA isolated from hypothalami of control and hypothermic rats (Table 1). In total,

Table 3
The 50 most abundantly expressed genes in hypothermic rats.

Tag sequence	Count of tags		Accession no	Corresponding gene
	Control	Hypothermia		
CATGAATCGGAGGCCAACCAGTAGAA	392	433		No match
CATGCTGCTGTACTAGACGTAGTTGC	58	328		No match
CATGGAATAATAAAACTATTTAAATT	106	122	BC098914	Heat shock protein 8
CATGGATCGCCCCCCACCTTACCACA	76	115		No match
CATGCTGCTGTGTGCATCGATAGCTA	15	107		No match
CATGCTGCTGTGTGCATCGATAGCAA	12	89		No match
CATGTTAATAAATGACCAGTTTACGT	57	72	NM_017202	Cytochrome c oxidase subunit IV isoform 1
CATGGATTCCGTGAAGGAACAACACC	32	44	BC069173	Ribosomal protein L37
CATGTTGACTTTATTACGATTTGTAT	31	44	NM_053865	Reticulon 1
CATGGAATGACCTGCAACTGTTAAAT	29	41	NM_001105730	Ribosomal protein S28
CATGATAATCACCTTAAGGCTTTTTG	21	41		No match
CATGAAGATGTGTTTTAAATGTCTAT	19	33	NM_024371	Solute carrier family 6, member 1
CATGCTGCTATCCGAGAGAATCCAGT	16	32	BC060561	Ribosomal protein L5
CATGCTGCTGTACTAGACGTAGTTGT	5	31		No match
CATGAGCTTGATTAAAATTGCTTGAC	16	28	NM_001106781	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12
CATGCATGTCAGGCTGCCTTTATCTT	12	25	BC127507	Ferritin, heavy polypeptide 1
CATGAATACGCAGAGGTGGTTTTCAC	9	24	BC086946	Transthyretin
CATGCGCCCGCAGGCAGCGTGCAGTC	11	23	NM_019217	Microtubule-associated protein 1b
CATGGGTCCAAGAGGAGCCAGTGCAG	12	22	BC098837	Nischarin
CATGGAGACAAGGCCAGCGTCAGAGA	11	22	BC087679	Angiotensinogen
CATGCTTCTGATAACATCATCAACTC	8	22	BC126087	Triosephosphate isomerase 1, mRNA
CATGGTGACCAAGTAGTATGTAGTTA	12	21		No match
CATGAAAAATGTACATCTTCATTCAT	10	19		No match
CATGATTATTGTATCCAATATTTTGT	6	17		No match
CATGCTACCCCTGGAAACCCAAATGG	8	16		No match
CATGAATCATCTTAGCTTAGCTTCCT	3	16	NM_017212	Microtubule-associated protein tau
CATGCTGCTGTGTGCATCGATAGAAG	0	16		No match
CATGGAAGAAGAATAAATGTGGGATT	6	15	NM_001106482	Plakophilin 4
CATGGTGAATTCGAGGTGTGGAATCT	6	14		No match
CATGCTAAGTAAAGGAACTCCTGCTT	3	14		No match
CATGGATCGATATTTTGATATGCGGT	3	14		No match
CATGCTGCTGTACTAGACGTAGTTGA	2	14		No match
CATGTTTCAGCATTTATTGTATATGC	5	13		No match
CATGAATCGGAAACTGGAACAAGGCA	5	12	NM_001007608	S-phase kinase-associated protein 1A
CATGAAGAAGGATCTCAGAATTTTGT	5	12		No match
CATGTTGTGAAATCTTTAGAGAACTT	4	12	NM_017309	Protein phosphatase 3, regulatory subunit B, alpha isoform
CATGACCTATATTGTGTTAACATCCT	3	12	NM_001013122	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2
CATGTTTTTATCTGCAGTTGTGTCTG	2	12		No match
CATGACCGTTATAAGAAAAGATATAT	4	11	NM_017068	Lysosomal-associated membrane protein 2
CATGCTGAGGAGGGGAAGGAGGAGGG	4	11	NM_057211	Kruppel-like factor 9
CATGCTTACCTCAGGATTCTTTAGTT	4	11	AF452728	Synaptogenesis-related mRNA sequence 7, 3′ untranslated region
CATGATAACACATAAAAAAAAAAAGG	4	11		No match
CATGTGATGTTTGCATTACTTTTAAC	4	11		No match
CATGTCCGTGCTAAGTTTGCAGCATT	3	11		No match
CATGCATTTGGCTATTGCTCAATTAA	2	11	NM_053532	Proteasome (prosome, macropain) subunit, beta type 7
CATGAAAACTCCAGCACCACATTCAT	3	11		No match
CATGCTCAGAGGTCCTACTGCCTCAG	3	10		No match
CATGACCCCGTCAGTCACCTCACTTA	3	9	NM_001139465	Transmembrane protein 59
CATGCCTTTGAGCAGAAAACTCTGAC	3	9		No match
CATGACGACATAGAAATAATTTAAAA	3	9		No match
CATGTTGAATTACTACTTCGCTGTAC	3	9		No match
CATGTATTGTATATGTTTATAGTATA	3	9		No match
CATGGTGTAATAAAGGTCGATAAGGT	3	9	NM_001105915	Acetylserotonin O-methyltransferase-like
CATGCAGGACCTTCGCAGTTCACCCT	3	9	NM_199207	Family with sequence similarity 21, member C

62,208 and 54,084 tags were collected from control and hypothermic hypothalami, respectively.

3.2. Differentially expressed tags between control and hypothermic rats

We focused our analysis on tags that were differentially expressed between control and hypothermic hypothalami. Although expression levels of most transcripts in both libraries were similar, 367 transcripts were differentially expressed at a statistically significant level—that is, 157 and 210 transcripts among them were expressed at a higher level in control and hypothermic hypothalami, respectively. Table 2 shows 50 transcripts overexpressed in the control hypothalamus compared with the hypothermic one, whereas Table 3 shows 50 transcripts overexpressed in the hypothermic hypothalamus compared with the control one. The tables are arranged in order of diminishing size. Of these genes, 102 were overexpressed by 5-fold in the control hypothalamus. And 149 were overexpressed by 5-fold in the hypothermic hypothalamus.

3.3. Validation of gene expression results by quantitative PCR

Table 4 documents the validation by quantitative PCR of 6 genes that were differentially expressed in control and hypothermic hypothalami. Results obtained by SuperSAGE and quantitative PCR were consistent, although levels of differences detected by the 2 methods were not exactly the same. In the control hypothalamus, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide expressed more than Purkinje cell protein 4.

4. Discussion

Understanding of molecular mechanisms underlying hypothermia is of primary importance in devising strategies to diagnose hypothermia. Techniques in transcriptomic analyses can be divided into two major classes. The first is based on hybridization of complementary nucleotide strands to immobilized target sequences. Microarray is a representative of this class. The second class involves sequencing and counting of transcripts. SAGE is an example.[13] SAGE is developed by Velculescu et al.[18] Briefly, a short tag of 13–15 bp representing each expressed sequence is excised from the cDNA. After sequencing thousands of tags, it is possible to count the number of each tag for transcripts and to describe the gene expression profile. Since the limited tag sequence size of only 13–15 bp was not always sufficient to unequivocally identify the gene from which the tag is derived,[11] SuperSAGE, a method for the isolation of 26 bp tag sequences by EcoP15I, is reported. EcoP15I dramatically improves conventional SAGE through reliable gene identifications.

Although in world wide use, microarrays still suffer from experiment-specific problems. The major drawback for microarray analyses is the fact that only genes whose sequences are on the chip, can be monitored. Therefore, gene discovery is not possible with this approach.[12] However, SuperSAGE detects unknown transcripts because it does not require prior knowledge of what is present in the sample. Furthermore, SuperSAGE permits the simultaneous detection and quantitation of the transcriptome from two intimately interacting organisms (e.g.: a host and a pathogen) without the physical separation of both.[12] Such a characteristic would be useful in forensic analyses of infectious cases. In addition, SuperSAGE is an absolute measure method, whereas microarray is a relative measure method. By recent new sequencing techniques, such as the emulsion-PCR-coupled pyrosequencing procedure (454 Life sciences, Branford, CT) and SOLiD sequencing technology (Applied Biosystems, Carlsbad, CA), sequencing will become cheaper.[13] In forensic pathology, SuperSAGE would thus be a promising method for gene expression analysis and biomarker detection.

The preoptic anterior hypothalamus is extremely important in thermoregulation.[19,20] The posterior hypothalamus integrates the central and peripheral temperature sensory signals.[21] Thus far, some hypothalamic reactions during hypothermia have been demonstrated. Rats in cold water caused a decrease in hypothalamic adrenaline and noradrenaline concentrations, and an increase in dihydroxyphenylacetic acid and homovanillic acid concentrations.[22] In the present study, the 50 most abundantly expressed genes in normal and hypothermic rats are annotated in Tables 2 and 3, respectively. Since the literature on these factors is limited, the mechanism of these responses is difficult to obtain at present. However, it is suggested that hypothermia results in decreasing cerebral blood flow, and declining neuronal metabolism.[2] Furthermore, the ability of the hypothalamus to regulate temperature is completely lost.[4] These findings may be relevant to the hypothalamic reaction. In the present study, hypothalamic transcript profiling in hypothermia is revealed. It is possible that hypothalamic gene expression could be useful for hypothalamic diagnosis. For daily forensic practices, it is unsuitable to apply all genes shown in the present study. However, some abundantly expressed genes could be useful for hypothermic diagnosis.

Table 4
Quantitative PCR to evaluate the validity of SuperSAGE.

	Control	Hypothermia
Abundantly expressed genes in control rats		
Neuroprotective protein 8:	25.6 ± 4.36	12.8 ± 3.97*
Purkinje cell protein 4:	0.226 ± 0.043	0.132 ± 0.052*
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide:	5.16 ± 0.503	2.50 ± 0.472*
Abundantly expressed genes in hypothermic rats		
Heat shock protein 8:	20.5 ± 3.13	27.6 ± 4.03*
Reticulon 1:	6.12 ± 1.65	11.4 ± 2.05*
Solute carrier family 6, member 1:	5.54 ± 1.12	9.39 ± 1.92*

Factor/β-actin.
Mean ± SD.
*: $p < 0.05$.

5. Conclusion

In forensic pathology, this is the first article to apply SuperSAGE to transcriptomic analysis and biomarker detections. Although expression levels of most transcripts were similar, 157 and 210 transcripts were expressed at a higher level in control and hypothermic hypothalami, respectively. Some abundantly expressed genes in the present study could be useful for hypothermic diagnosis.

Conflict of interest
None declared.

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Ethical approval
Obtained from institute ethics committee.

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References

1. Stocks JM, Taylor NAS, Tipton MJ, Greenleaf JE. Human physiological responses to cold exposure. *Aviat Space Environ Med* 2004;**75**:444–57.
2. Ulrich AS, Rathlev NK. Hypothermia and localized cold injuries. *Emerg Med Clin North Am* 2004;**22**:281–98.
3. Paton BC. Accidental hypothermia. *Pharmacol Ther* 1983;**22**:331–77.
4. DiMaio VJ, DiMaio D. Hypothermia. In: *Forensic pathology*. 2nd ed. Boca Raton: CBC Press; 2001. p. 428–34.
5. Hirvonen J. Necropsy findings in fatal hypothermia cases. *Forensic Sci* 1976;**8**:155–64.
6. Preuss J, Dettmeyer R, Lignitz E, Madea B. Fatty degeneration in renal tubule epithelium in accidental hypothermia victims. *Forensic Sci Int* 2004;**141**:131–5.
7. Preuss J, Dettmeyer S, Poster E, Lignitz E, Madea B. The expression of heat shock protein 70 in kidneys in cases of death due to hypothermia. *Forensic Sci Int* 2008;**176**:248–52.
8. Saukko P, Knight B. Injury caused by cold: hypothermia. In: *Knight's forensic pathology*. 3rd ed. , London: Arnold; 2004. p. 414–20.
9. Wedin B, Vanggaard L, Hirvonen J. Paradoxical undressing in fatal hypothermia. *J Forens Sci* 1979;**24**:543–53.
10. Matsumura H, Nirasawa S, Terauchi R. Transcript profiling in rice (*Oryza sativa* L.) seedings using serial analysis of gene expression (SAGE). *Plant J* 1999;**20**:719–26.
11. Matsumura H, Reich S, Ito A, Saitoh H, Kamoun S, Winter P, et al. Gene expression analysis of plant host–pathogen interactions by SuperSAGE. *Proc Natl Acad Sci U S* 2003;**A 100**:15718–23.
12. Matsumura H, Reuter M, Kruger DH, Winter P, Kahl G, Terauchi R. SuperSAGE. *Methods Mol Biol* 2008;**387**:55–70.
13. Terauchi R, Matsumura H, Kruger DH, Kahl G. SuperSAGE: the most advanced transcriptome technology for functional genomics. In: Kahl G, Meksem K, editors. *Handbook of plant functional genomics*. Weinheim: Wiley; 2008. p. 37–54.
14. Okuda C, Saito A, Miyazaki M, Kuriyama K. Alteration of the turnover of dopamine and 5-hydroxytryptamine in rat brain associated with hypothermia. *Pharmacol Biochem Behav* 1986;**24**:79–83.
15. Glowinski J, Iversen LL. Regional studies of catecholamine in the rat brain-I. The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]DOPA in various regions of the brain. *J Neurochem* 1966;**13**:655–69.
16. Meisel A, Bickle T, Kruger DH, Schroeder C. Type III restriction enzymes need two inversely oriented recognition sites for DNA cleavage. *Nature* 1992;**355**:467–9.
17. Man MZ, Wang X, Wang Y. POWER_SAGE: comparing statistical test of SAGE experiments. *Bioinformatics* 2000;**16**:953–9.
18. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995;**270**:484–7.
19. Boulant JA. Hypothalamic neurons. Mechanisms of sensitivity to temperature. *Ann N Y Acad Sci* 1998;**856**:108–15.
20. Romanovsky AA. Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. *Am J Physiol Regul Integr Comp Physiol* 2007;**292**:R37–46.
21. Guyton AC, Hall JE. Body temperature, temperature regulation, and fever. In: *Textbook of medical physiology*. Philadelphia: Elsevier; 2006. p. 889–901.
22. Roth KA, Mefford IM, Barchas JD. Epinephrine, norepinephrine, dopamine and serotonin: differential effects of acute and chronic stress on regional brain amines. *Brain Res* 1982;**239**:417–24.